Project Report
Arizona Iceberg Lettuce Research Council

For period: July 2005 through June 2006

Title: Determination of *Mirafiori Lettuce Virus* and *Lettuce Big Vein Virus* Incidence and Genetic Variability in Big Vein Infected Lettuce.

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INTRODUCTION

Big vein is a viral disease of lettuce (*Lactuca sativa* L.) transmitted by the soil-borne fungus *Olpidium brassicae* (Jagger and Chandler 1934). Symptoms of big vein include chlorosis surrounding the vascular bundles in the leaf and increased stiffness of the leaves that disrupts normal head development, resulting in plants that have a bushy appearance. Reduced frequency of head formation is the primary source of economic damage resulting from big vein disease. Big vein is most prevalent in cool wet soils (Campbell and Grogan 1963, Westerlund *et al.* 1978a, 1978b), and increases with continuous lettuce production without rotation. Consequently, big vein consistently occurs at high levels during spring production in California's coastal growing districts, and during winter production in Arizona.

Effective long-term control of big vein disease is best accomplished through genetic resistance, and is important for sustainable production of quality lettuce. Complete resistance to big vein has only been identified in accessions of *L. virosa* L. (Bos and Huijberts 1990), but this resistance has not been introgressed into lettuce cultivars to date. Among cultivated lettuce, partially resistant cultivars are available that have a reduced frequency of symptomatic plants and/or symptom expression that is delayed until plants reach market maturity (Ryder and Robinson 1995). This type of resistance has greatly improved marketable yields in fields infested with big vein (Ryder 1979). Progress in increasing the level of partial resistance has been slow, primarily because of a lack of information regarding the pathogen, the unknown inheritance of resistance, and the large influence that environmental conditions have on symptom expression.

Although big vein disease has impacted lettuce production for many years, the causal agent, *Mirafiori lettuce big-vein virus* (MLBVV) (genus *Ophiovirus*), formerly known as *Mirafiori lettuce virus*, was only recently identified (Lot et al., 2002; Roggero et al., 2003a). Another virus, *Lettuce big-vein associated virus* (LBVaV) (genus *Varicosavirus*), formerly known as *Lettuce big vein virus*, was previously found associated with big vein disease, but a causative relationship was never confirmed (Huijberts et al. 1990; Vetten et al. 1987). Interestingly, many studies have demonstrated that plants exhibiting big vein symptoms were frequently coinfected with both viruses, suggesting LBVaV may also contribute in some manner to disease (Roggero et al., 2003b; Navarro et al., 2004, 2005).

Understanding the distribution of MLBVV and LBVaV in Arizona and California and the genetic relationships among virus isolates affecting western production is important for developing control methods suitable for production conditions in the western U.S. Additionally, knowledge of the virus(es) responsible for big vein disease provides an opportunity to develop more effective methods of screening for resistance, and identification of plants not only with reduced symptom expression, but also with reduced virus incidence. Coupling these methods will facilitate more reliable resistance testing than those used previously by lettuce breeders, and ultimately may lead to improved quality and performance of resistant lettuce.

PREVIOUS ACCOMPLISHMENTS

Research conducted last year demonstrated that isolates of MLBVV from California and Arizona and of LBVaV from California were closely related genetically to international isolates. Consequently any resistance sources developed should be useful in all lettuce production regions where big vein occurs. Our research substantiated previous reports (Roggero et al. 2003a,b; Navarro et al. 2004), showing a strong dependence between big vein symptom expression and MLBVV presence in Yuma grown lettuce. In addition our research also demonstrated that symptomatic and asymptomatic plants from both resistant and susceptible *L. sativa* cultivars could accumulate MLBVV and LBVaV (although we did not identify any LBVaV in Arizona lettuce during the 2004-2005 season). Among wild relatives of lettuce, only accessions of *Lactuca virosa* have demonstrated a complete lack of symptom expression in inoculated trials (Bos and Huijberts, 1990). *Lactuca virosa* accession IVT280 was identified as 100% asymptomatic in our inoculated greenhouse trials. Analysis by reverse transcription-polymerase chain reaction (RT-PCR) demonstrated no amplification of MLBVV and LBVaV, indicating apparent immunity in this accession (Hayes et al., 2006).

OBJECTIVES

1. Develop Real-Time RT-PCR for measuring the concentration of MLBVV and LBVaV in lettuce germplasm as a tool to determine the level of virus resistance in *Lactuca* species.

2. Determine LBVaV and MLBVV accumulation in BC_1F_3 progeny of *L. sativa x L. virosa* hybrids, *L. virosa* accessions, tolerant *L. sativa* cultivars, and susceptible *L. sativa* cultivars using Real-Time RT-PCR.

3. Test *L. virosa x L. sativa* F_1 hybrids for infection by both MLBVV and LBVaV using traditional RT-PCR to determine whether apparent complete resistance (lack of virus accumulation) in *L. virosa* is dominant to susceptibility in *L. sativa.*

4. Test additional lettuce samples from Yuma area to determine if LBVaV can be detected or if this virus is simply not able to establish under Yuma conditions.

MATERIALS AND METHODS

Collection of field samples and classification of symptom severity. Lettuce leaf samples were collected for virus RNA isolation from 13 field sites in the Yuma, Arizona production area. Five to nine plants per site were sampled by collecting one complete leaf per plant. Leaves were stored on ice, brought into the lab and classified as healthy, mild, moderate, or severe for big vein symptoms using a disease severity scale. Lettuce tissue was sampled (100 mg per sample), lyophilized, and stored at -80°C prior to RNA extraction.

Greenhouse testing of big vein resistance. Greenhouse experiments were performed to compare big vein resistance among *L. sativa* cultivars and *L. virosa*, following the protocol of Ryder and Robinson (1995). Seedlings were germinated in a sand-field soil potting mix and grown for three weeks. A suspension of *O. brassicae* zoospores was prepared by macerating the roots of big vein symptomatic plants in water. The seedlings were inoculated by watering the zoospore suspension into the seedling pots twice per inoculation, with inoculation intervals separated by 1 day. Each seedling was subsequently transplanted into an 8 cm pot containing field soil. Plants were grown in a greenhouse maintained at 18°C, and the percentage of symptomatic plants was recorded after 6 to 8 weeks of growth. Tissue was sampled from asymptomatic and symptomatic plants for RNA or protein.

RT-PCR. Tissue samples were ground in liquid nitrogen, and total RNA extracted using the Qiagen RNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturer's recommendations. RNA extracts were stored at -80°C. MLBVV and LBVaV coat protein RT-PCR primer pairs were designed from published MLBVV and LBVaV sequences. RNA extracts (as well as positive and negative controls, and reagent blanks) were reverse-transcribed, and cDNA was amplified by polymerase chain reaction (RT-PCR). RT-PCR reactions were electrophoresed on 1% agarose stained with ethidium bromide, and the presence or absence of the target band determined. All samples from which MLBVV or LBVaV RNA did not amplify were re-analyzed using a different primer pair, to rule out the occurrence of false negatives, and ultimately re-tested with molecular probes for LBVaV and MLBVV by nucleic acid hybridization. For additional information on techniques see our recent Plant Disease paper (Hayes et al. 2006).

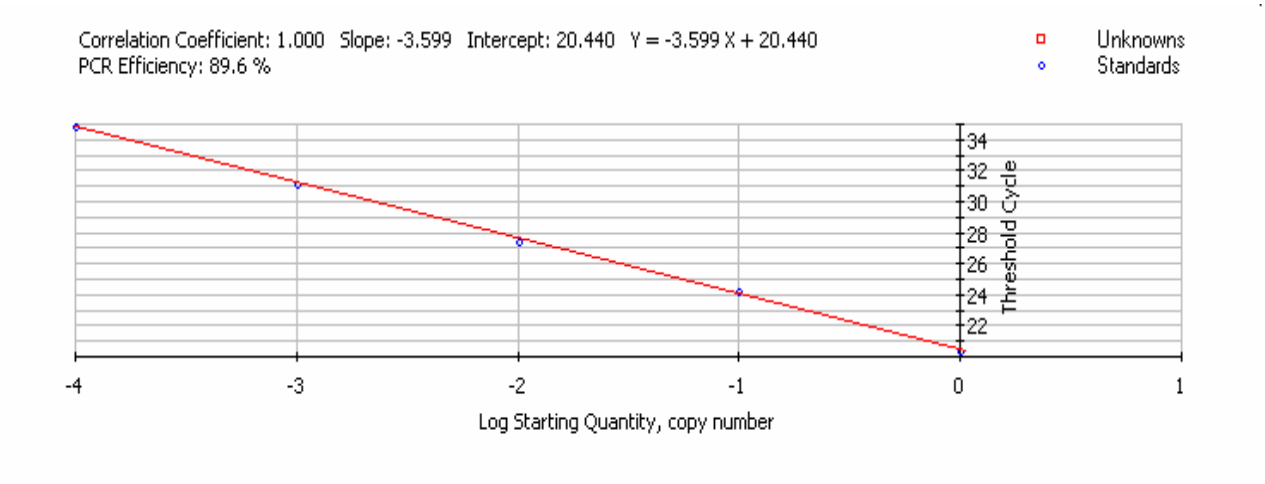
RESULTS AND DISCUSSION

Objective 1. Develop Real-Time RT-PCR for measuring the concentration of MLBVV and LBVaV in lettuce germplasm as a tool to determine the level of virus resistance in *Lactuca* species.

Primers were developed for use in determining the specific amount of MLBVV in lettuce tissue by real time RT-PCR. Since it is now clear that LBVaV is not involved in symptom induction, no efforts have been made to consider LBVaV levels. In addition, only a very small proportion of plants in the Yuma production region appear to have LBVaV (see Objective 4 results). We have developed a real time RT-PCR method to quantify MLBVV that quantitatively and selectively amplifies MLBVV from infected lettuce tissue (Figure 1). This method will be used for completion of Objectives 2 and 3 over the next few months. In addition, a source of

antiserum for detection of MLBVV was identified this spring, and this antiserum is also being tested for its ability to accurately quantify levels of MLBVV in infected tissues of cultivated (*L. sativa*) and wild (other *Lactuca* species) lettuces, by comparison with the other methods of detection available in our laboratory (data not shown).

Figure 1. Graph of threshold cycle (C_t) vs starting amount of template in quantitative (real-time) RT-PCR of MLBVV. The location of points on the curve indicates the primers used should be accurate in calculating the amount of MLBVV in samples by comparing their C_t values to this graph. Efficiency indicates number of doublings per cycle (theoretical max is 100% = 1 doubling)



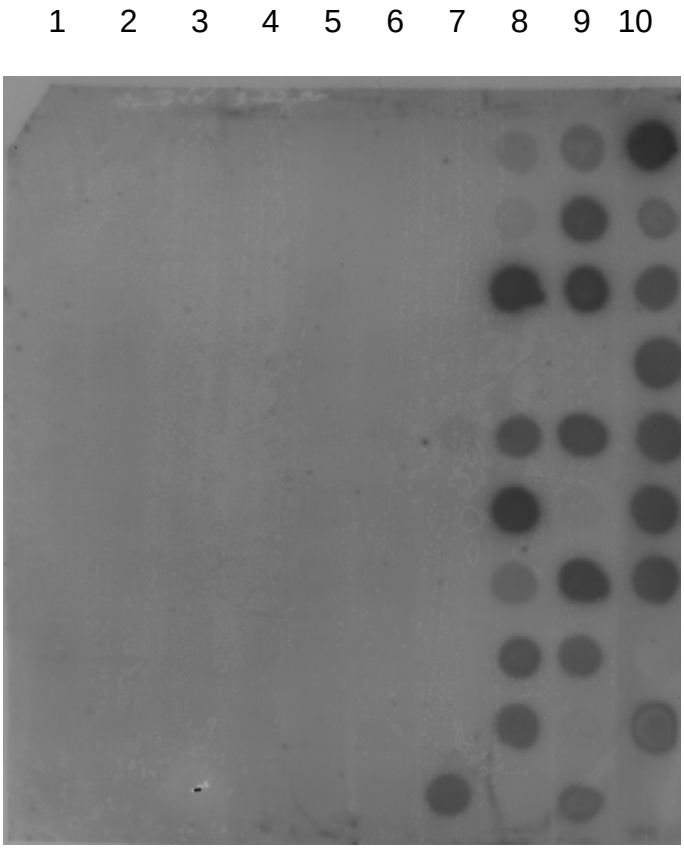
Objectives 2 and 3: Activity on these objectives has been delayed by the discovery this winter that the putative immune *L. virosa* accession, IVT280 may not be completely immune to replication of MLBVV, the virus that causes lettuce big vein disease. Nucleic acid spot hybridization (NASH) was used to rapidly test large numbers of *L. virosa* accession in a greenhouse trial for immunity. Numerous initial tests, including results shown below (Figure 2), suggested complete immunity of IVT280 to MLBVV; whereas other *L. virosa* accessions allowed accumulation of MLBVV.

Additional tests later identified a number of *L. virosa* accessions, including IVT280, previously believed to be immune, that permitted varying levels of MLBVV accumulation by either NASH (data not shown) or RT-PCR (Figure 3). This was quite unexpected based on limited results obtained during the first year of this study (see 2005 annual report) and early results this year (Figure 2), which suggested IVT280 was immune. These results indicated that many of the plants originally believed to be immune, may actually accumulate some level of MLBVV under certain conditions. It is possible that accumulation in these varieties is quite low, but to date we have focused primarily on determining if accumulation occurs rather than how much accumulation occurs. Since virus accumulation in the parental material (*L. virosa*) may impact our ability to obtain high levels of resistance in progeny, we have spent considerable effort this spring to clarify the nature of the accumulation and how best to detect it in both parental and progeny material. Recently obtained antiserum against MLBVV may facilitate less expensive

quantification of virus than with real-time RT-PCR, however, we still have some questions about background levels in serological detection that may interfere with determining MLBVV accumulation in partially resistant wild *Lactuca* species. Quantitative RT-PCR may still be necessary to clearly differentiate immunity from partial resistance.

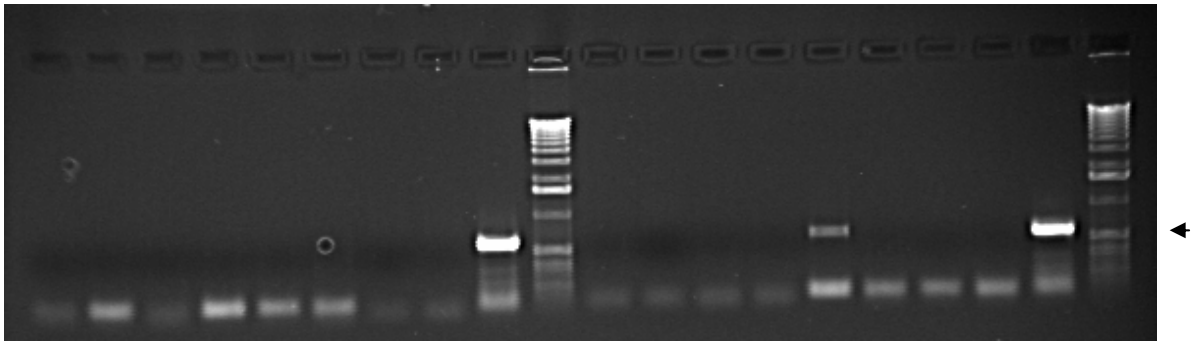
Thirty-three *L. virosa* x *L. sativa* F_1 hybrids were tested for symptom expression and virus accumulation using RT-PCR and NASH. No symptoms were observed in these hybrids (data not shown). Low levels of virus accumulation were detected in 25% of the test plants, while no virus was detected in the remaining plants (data not shown). This phenotype is similar to what has been observed in *L. virosa* accessions, and suggest that the *L. virosa* resistance phenotype is dominant to susceptibility in lettuce. Further testing with real time RT-PCR in advanced generation progeny is needed to confirm this hypothesis.

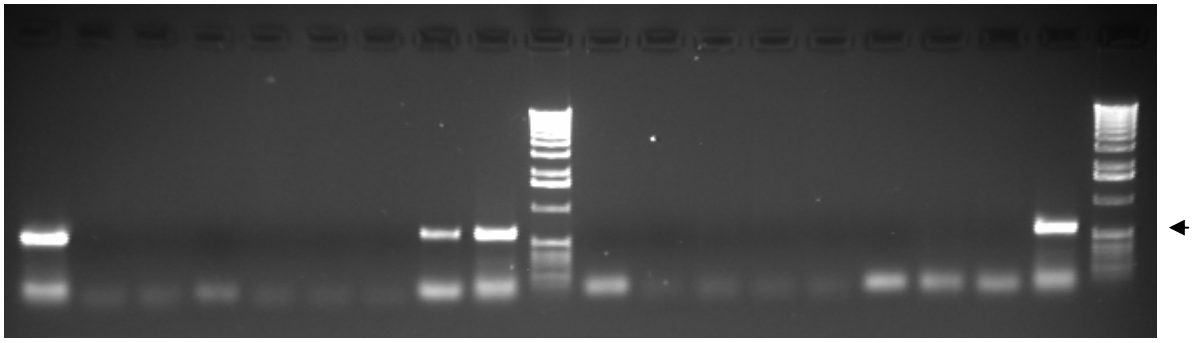
Figure 2. Nucleic acid spot hybridization indicating the presence or absence of MLBVV in IVT280 and other *L. virosa* accessions as detected using a probe to the coat protein gene of MLBVV[a], and suggesting immunity in IVT280.



[a] Columns are labeled left to right and spots are numbered in increasing order from top to bottom. Columns 1-6 = IVT280; Column 7 spots 1-10 = IVT280, spot 11 = CGN16272; Column 8-9 and Column 10 spots 1 and 2 = other *L. virosa* accessions. Column10 spots 3-5= Salinas88, spots 6-8 = Pacific, spot 9 = healthy lettuce, spot10 = symptomatic GL65, spot11=blank.

Figure 3. RT-PCR using MLBVV coat protein primers indicating detection of MLBVV in IVT280 samples 49, 54, and 61, previously believed to be immune[b].





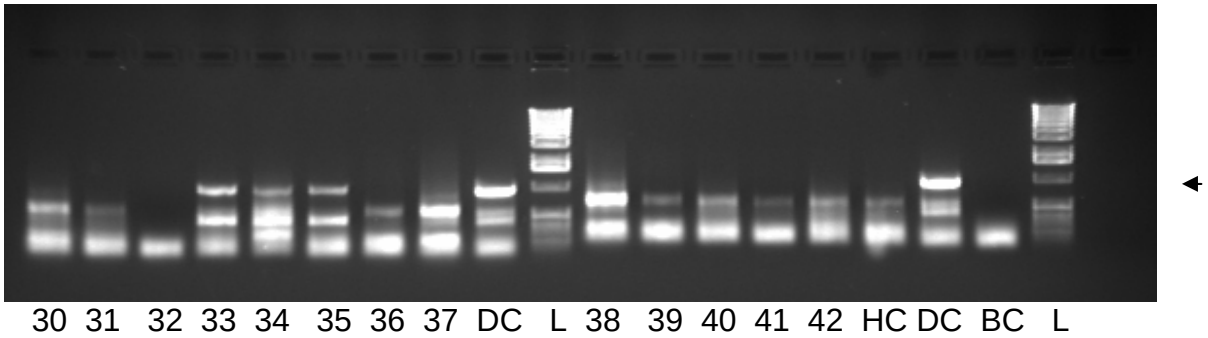
[b] Primers amplify a 590 bp product. Arrowhead indicates location of relevant band. L = DNA size standard, DC = Disease check (positive control), healthy and blank checks not shown on this section of gel but did not contain bands.

Objective 4. Test additional lettuce samples from Yuma area to determine if LBVaV can be detected or if this virus is simply not able to establish under Yuma conditions.

During the 2004-2005 growing season, we only detected MLBVV in Yuma area lettuce, but not LBVaV. In all other areas of the world where big vein disease is found and where attempts have been made to identify these viruses, both have been found together in the majority of plants tested. Yuma is the only region of the world to date where attempts have been made to identify these viruses that LBVaV has not been found associated with big vein disease. Although MLBVV is the causative agent and LBVaV is merely associated, it is highly unusual not to find LBVaV associated as it is transmitted by the same vector, *O. brassicae*, and routinely co-infects plants with MLBVV. Importantly, parallel studies conducted in the Salinas Valley of California identified co-infection of LBVaV and MLBVV in 83% of plants, which was a significant association compared to random distribution of each virus (data not shown). To determine conclusively whether LBVaV was present in Yuma soils, plants exhibiting big vein symptoms were collected from fields throughout the Yuma growing region in Arizona and tested by RT-

PCR for the presence of LBVaV in January 2006. Among plants sampled from the Yuma area this year, all those exhibiting big vein symptoms, as well as some that had not yet developed symptoms contained MLBVV, as expected. LBVaV was found in Yuma samples this year, confirming the presence of LBVaV in Yuma area soils. Interestingly, of 42 plants sampled from 13 fields exhibiting big vein symptoms, only 3 plants (7%) were positive for LBVaV, suggesting incidence of LBVaV in Yuma may be quite low compared to MLBVV. A photo of the gel documenting LBVaV incidence is shown below (Figure 4). Since LBVaV is not believed to play a role in development of big vein disease and does not interfere with transmission of MLBVV by *O. brassicae*, this low level is more of a curiosity than an issue of agricultural significance.

Figure 4. Yuma big-vein lettuce samples collected in January 2006 showing the presence of positive bands for LBVaV in samples 33, 34 and 35 [c].



[c] L = DNA size standard, DC = Disease check (positive control), HC = Healthy check (negative control), BC = blank check (no DNA added to RT-PCR reaction, only primers present). Arrowhead indicates location of relevant band (top band, 385 bp).

SUMMARY

Research in big vein disease control has been limited by a lack of knowledge regarding the pathogen. Research conducted during the first year of this project confirmed previous reports (Roggero et al. 2003; Navarro et al. 2004), showing a strong dependence between big vein symptom expression and MLBVV presence in Yuma grown lettuce, further illustrating the causative role of MLBVV in big vein disease (Hayes et al., 2006). Furthermore our work demonstrated that big vein disease in Arizona and California production areas results from MLBVV isolates nearly identical to isolates from other parts of the world. Consequently resistance sources identified here and elsewhere should be universally applicable for control of big vein disease of lettuce. We have also shown that symptomatic and asymptomatic plants from both resistant and susceptible *L. sativa* cultivars can accumulate both MLBVV and LBVaV, and that both viruses are present in Yuma area soils, although LBVaV incidence is apparently quite low in these soils.

Among wild relatives of lettuce, only accessions of *Lactuca virosa* have demonstrated a complete lack of symptom expression in inoculated trials (Bos and Huijberts, 1990). *Lactuca*

virosa accession IVT280 was identified as 100% asymptomatic in the inoculated greenhouse trials reported here. Analysis by RT-PCR demonstrated no viral amplification in initial studies, indicating apparent immunity in this accession. More extensive testing, however, has demonstrated that some MLBVV accumulation can occur in IVT280. The extent and conditions under which this occurs are still being examined. Breeding efforts using big vein immunity from IVT280 are being pursued (Hayes et al., 2004). The development of quantitative (real-time) RT-PCR, and recent availability of MLBVV-specific antiserum will greatly improve our ability to monitor virus levels in breeding material, and should facilitate development of diverse lettuce cultivars with greatly improved big vein resistance, derived from *L. virosa*.

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